

Soluble Epoxide Hydrolase Inhibition Protects the Kidney from Hypertension-Induced Damage

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Abstract. Epoxyeicosatrienoic acids (EET) have antihypertensive and anti-inflammatory properties and play a role in the maintenance of renal vascular function. A novel approach to increase EET levels is to inhibit epoxide hydrolase enzymes that are responsible for conversion of biologically active EET to dihydroxyeicosatrienoic acids (DHET). We hypothesized that soluble epoxide hydrolase (SEH) inhibition would improve renal vascular function and ameliorate hypertension induced renal damage. Chronic administration of the specific SEH inhibitor 1-cyclohexyl-3-dodecylurea (CDU, 3 mg/d) for 10 d lowered BP in angiotensin hypertensive rats. The contribution of renal vascular SEH to afferent arteriolar function in angiotensin hypertension was also assessed. SEH protein ex-

pression was increased in renal microvessels from hypertensive rats. Although CDU did not change afferent arteriolar responsiveness to angiotensin in normotensive animals, CDU treatment significantly attenuated afferent arteriolar diameter responses to angiotensin in hypertensive kidneys from 51% \pm 8% to 28% \pm 7%. Protection of the renal vasculature and glomerulus during chronic CDU administration was demonstrated by histology. Urinary albumin excretion, an index of renal damage, was also lower in CDU-treated hypertensive rats. These data demonstrate that SEH inhibition has antihypertensive and renal vascular protective effects in angiotensin hypertension and suggests that SEH inhibitors may be a useful therapeutic intervention for cardiovascular diseases.

Cytochrome P450 metabolites are now recognized as major regulators of renal and cardiovascular function (1–5). Epoxyeicosatrienoic acids (EET) are cytochrome P450 products of arachidonic acid that can be further metabolized by epoxide hydrolases to form the corresponding dihydroxyeicosatrienoic acids (DHET). Hydrolysis of the EET to the corresponding DHET generally is regarded as one mechanism whereby the biologic effects of the EET are attenuated or eliminated (1–5). In any case, EET have effects on vascular cells, as well as, renal tubular ion and water transport and have been implicated in the control of BP. Interest in epoxygenase metabolites was heightened by their identification as endothelium-derived hyperpolarizing factors (EDHF) (6–8). Emerging evidence also strongly suggest that EET have protective cardiovascular effects beyond their actions as EDHF. Intriguingly, EET possess anti-inflammatory actions and a recent study has demonstrated that 11,12-EET antagonizes the development of vascular lesions by acting as a local antimigratory factor for vascular smooth muscle cells (9–11). 11,12-EET also possesses cardio-

vascular protective fibrinolytic activity by inducing tissue-type plasminogen activator (10). Taken as a whole, there is ample evidence to support the hypothesis that increasing EET levels would have beneficial effects on renal and cardiovascular function in disease states.

Others and we have found that decreased renal epoxygenase levels are associated with the development of hypertension (1,5,12,13). One approach to increase EET levels chronically is to inhibit the soluble epoxide hydrolase (SEH) enzyme that metabolizes EET to their corresponding DHET. Fortunately, this enzyme represents a single known and highly conserved gene product with over 90% homology between humans, rats, and mice and can be selectively inhibited by a variety of urea, carbamate, and amide derivatives *in vivo* and *in vitro* (14–17). On the basis of the anti-inflammatory and antihypertensive properties of EET, we hypothesized that SEH inhibition offers a novel target for the treatment of end organ damage associated with renal and cardiovascular diseases.

Materials and Methods

Animals

The Medical College of Georgia Animal Care and Use Committee approved the experimental procedures. Male Sprague-Dawley rats were divided into three experimental groups; the first group was subjected to sham surgery, the second group received angiotensin and vehicle treatment, and the third group received angiotensin and 1-cyclohexyl-3-dodecylurea (CDU, a.k.a. NCND). Angiotensin was in-

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fused at a continuous rate with a minipump (65 ng/min) as described previously (14). On day 10, CDU treatment was started. CDU was added to corn oil (3 mg/100 μ l) and warmed to 30°C, vigorously vortexed, and sonicated until the CDU was homogeneously suspended. CDU was administered once daily (3 mg/d intraperitoneally) for 10 d, and on the last day of treatment, rats were placed in metabolic cages to facilitate 24-h urine collection.

Measurement of BP

Telemetry transmitters (Data Sciences, St. Paul, MN) were implanted and data collected as described previously (18). Minute-to-minute 24-h measurement of mean arterial pressure as well as the heart rate was obtained. The Biotelemetry Core at the Medical College of Georgia provided assistance with these studies.

Measurement of Oxylipids

The urinary levels of arachidonic and linoleic acid metabolites were measured as described previously (19). Animals were housed in metabolic cages that separated urine from food and feces. Urine was collected in a tube containing 5 mg of triphenylphosphine and cooled with dry ice. Blood was collected into tubes containing heparin and centrifuged to obtain plasma. Samples were stored at –80°C until assayed. Sample aliquots (urine = 4 ml; plasma = 250 μ l) were removed from thawed samples. These aliquots were then spiked with analytical surrogates. Urine samples were extracted twice with 2 ml ethyl acetate, and plasma samples were extracted twice with 1 ml ethyl acetate. The combined organic extracts were dried under nitrogen and dissolved in 100 μ l of methanol and spiked with internal standards. A 10- μ l aliquot of the methanolic extract was then separated by reverse phase HPLC and analyzed by negative-mode electrospray ionization and tandem mass spectroscopy as described previously (19). EET and DHET arachidonic acid metabolites and epoxyoctadecanoic acid (EPOME) and dihydroxyoctadecanoic acid (DHOME) linoleic acid metabolites were assessed.

Assessment of CDU Levels

Urine and plasma samples were collected after the 10-d treatment period. CDU and CDU metabolites were measured as described previously (20). In the case of urine samples, a 500- μ l aliquot was spiked with 50 μ l ([Final] = 500 ng/ml) of 1-cyclohexyl-3-tetradecylurea as an internal standard. For plasma samples, a 100- μ l aliquot was diluted with 200 μ l of distilled water and mixed by vortex before internal standard addition. Samples were then mixed, followed by extraction with 500 μ l of ethyl acetate. The samples were centrifuged at 6000 rpm for 5 min, and the ethyl acetate was collected. The organic extraction was repeated and the extracts were combined, evaporated to dryness under dry N₂ gas, reconstituted in 50 μ l of MeOH, and a 5- μ l aliquot was analyzed. Mass spectrometry analysis was performed with positive mode electrospray ionization on a Micromass Ultima triple quadrupole mass spectrometer with multireaction monitoring (20).

Isolation of Renal Microvessels

Renal microvessels were isolated according to a method described previously (6). Briefly, the kidneys were infused with a physiologic salt solution containing 1% Evans blue, and the renal microvessels were separated from the rest of the cortex with the aid of sequential sieving, a digestion period and collection under a stereomicroscope. Renal microvessels were collected, quickly frozen in liquid N₂, and kept at –80°C in a freezer until assayed for protein levels.

Western Blot Analysis

Renal microvessels and kidney cortical samples were harvested and homogenized for Western blot analysis as described previously (12). Samples were separated by electrophoresis on a 10% stacking Tris-glycine gel, and proteins were transferred electrophoretically to a polyvinylidene fluoride membrane. The primary antibodies used were rabbit anti-mouse SEH antibody (Dr. Hammock) and rabbit anti-rat microsomal epoxide hydrolase (MEH) antibody (Drs. Oesch and Arand, University of Mainz). The blots were then washed and incubated with the goat anti-rabbit secondary antibody conjugated to horseradish peroxidase. Detection was accomplished with enhanced chemiluminescence Western blot test (ECL). Band intensity was measured densitometrically and the values were factored for β -actin.

Renal Microvascular Responses

In vitro perfused juxtamedullary nephron preparation was used to assess renal microvascular reactivity as described previously (6). The isolated kidney was perfused with a reconstituted red blood cell containing solution and renal artery perfusion pressure was set to 100 mmHg. After a 20-min equilibration period, an afferent arteriole was chosen for study and baseline diameter was measured. After control diameter measurements, angiotensin (0.1 to 10 nM) was delivered by superfusion and a concentration response curve was obtained. Each vessel then underwent a 5-min recovery period before being exposed to 1 μ M CDU for 10 min. Angiotensin containing solution was reintroduced after SEH inhibition and the afferent arteriolar response was reassessed.

Evaluation of Renal Vascular and Glomerular Injury

At the end of the 10-d CDU treatment period, kidneys were immediately fixed in 10% buffered formalin solution and embedded in paraffin for light microscopic evaluation. Sections were cut at a thickness of 2 to 3 μ m and stained with hematoxylin-eosin, periodic acid-Schiff reagent, and periodic acid-methenamine silver. For semi-quantitative evaluation, Dr. Yamamoto graded histologic sections for renal injury in a blind fashion. Histologic sections were evaluated for mesangial proliferation, mesangial expansion, interstitial mononuclear cell infiltration, and arteriolar thickening. A grade was given for each section evaluated and the following grades applied for the extent of renal injury: +1 = very mild; +2 = mild; +3 = moderate; and +4 = severe. Histologic sections were evaluated from five animals in each group and an average score for each category determined.

Immunoreactive collagen type IV was assessed in fixed kidney slices. The sections were incubated in 3% (vol/vol) of hydrogen peroxide in PBS containing 0.1% Tween-20 for 15 min to quench endogenous peroxidase activity. The sections were subsequently incubated in goat serum for 1 h at room temperature to block nonspecific binding. Next, the rabbit polyclonal collagen type IV (1:50, Santa Cruz Chemicals) was incubated overnight at room temperature. Peroxidase-conjugated donkey anti-rabbit IgG (1:250) was then applied for 30 min, and the sections were washed with PBS. Chromogen 3,3'-diaminobenzidine was added to the sections for 6 min, washed with distilled water, and counterstained with hematoxylin. The sections were then examined by light microscopy for collagen IV positive staining.

Urinary albumin was assessed as another index for renal injury. Albumin levels were measured by a competitive enzyme immunoassay (Nephra; Exocell).

Statistical Analyses

All data are presented as mean \pm SEM. The significance of differences between groups for the BP and renal vascular data were

evaluated with an ANOVA for repeated measures followed by a Duncan's multiple-range post hoc tests. An unpaired *t* test was applied to compare the SEH and MEH protein levels, histologic grading, and urinary oxylipid levels. A *P* value of <0.05 was considered significant.

Results

We assessed BP and heart rate by telemetry; the results are presented in Figure 1. Consistent with previous reports (12,14,21), BP was significantly increased in angiotensin-infused rats over the initial 10-d infusion period. Administration of the SEH inhibitor CDU was started on day 10 and was continued for 10 d. CDU treatment significantly (*P* < 0.05) lowered BP in angiotensin-infused rats. BP in angiotensin hypertensive rats treated with vehicle averaged 156 ± 1 mmHg at night and 140 ± 3 mmHg during the day from days 15 to 20. Angiotensin-infused animals treated with CDU had a BP that

averaged 139 ± 3 mmHg at night and 123 ± 4 mmHg during the day over the same 5-d period.

Heart rate averaged 385 ± 6 bpm during the day and 419 ± 3 bpm at night before the start of angiotensin infusion. As expected, heart rate declined initially after the start of the angiotensin infusion. Heart rate reached a nadir and averaged 353 ± 11 bpm during the day and 383 ± 14 bpm at night 2 d after the initiation of angiotensin administration. Heart rate returned to levels similar to control values by day 10 of angiotensin infusion. CDU or corn oil did not alter heart rate over the 10-d treatment period. Heart rate averaged 366 ± 19 bpm during the day and 420 ± 19 bpm at nighttime after 10 d of CDU treatment and was not significantly different from values in vehicle-treated rats.

Plasma and urine CDU and metabolite levels were measured to confirm proper epoxide hydrolase inhibitor treatment. A CDU background signal of unknown origin was detected in control animals (plasma: 1.2 ± 0.2 ng/ml, urine 0.1 ± 0.06 ng/ml) and vehicle-treated angiotensin hypertensive rats (plasma: 1.4 ± 0.3 ng/ml, urine 0.1 ± 0.04 ng/ml). Metabolites of CDU were below detectable limits in the nontreated groups and the aldehyde metabolite, 1-cyclohexyl-3-(12-oxo-dodecyl)-urea (CODU), was below detectable limits in all groups. Angiotensin hypertensive animals treated for 10 d with CDU had significant levels of CDU in the plasma and urine. Plasma CDU levels were 23.1 ± 4.1 ng/ml and the hydroxylic metabolite, 1-cyclohexyl-3-(12-hydroxy-dodecyl)-urea (CHDU) and carboxylic metabolite, 12-(3-cyclohexyl-ureido)-dodecanoic acid (CUDA) averaged 32.9 ± 10.5 ng/ml and 4.1 ± 1.5 ng/ml, respectively, in CDU-treated animals. Ten days of CDU treatment resulted in 10.6 ± 2.4 ng/ml of CDU in the urine and detectable levels of CHDU (0.7 ± 0.3 ng/ml) and CUDA (0.3 ± 0.1 ng/ml).

Plasma arachidonic and linoleic acid metabolite levels at the end of the 20-d angiotensin infusion period are presented in Table 1. The plasma arachidonic and linoleic acid metabolite levels reflect the combination of soluble components, those bound to hydrophobic surfaces, and those incorporated into circulating phospholipids and triglycerides. Interestingly, we observed increased plasma EPOME and decreased DHOME in angiotensin-infused CDU-treated compared with angiotensin-infused corn oil-treated rats. EPOME increased by 126% and DHOME decreased by 60% in angiotensin-infused rats treated with CDU. On the other hand, urinary arachidonic and linoleic acid metabolite levels appear to be a better reflection of the prevailing kidney SEH levels (Table 2). The EPOME:DHOME and EET:DHET ratios were decreased by 50% in the angiotensin-infused group compared with the control group. CDU treatment increased these ratios but had a greater effect on the EET:DHET ratio. EPOME excretion rate increased significantly by 49% in angiotensin-infused CDU-treated rats compared with vehicle-treated angiotensin-infused rats. Unlike plasma levels, the urinary EET and DHET excretion rates had changes consistent with the level of kidney SEH protein expression and SEH inhibition. Although total urinary DHET were not significantly altered, 14,15-DHET levels averaged 2.7 ± 1.5 pmol/d in control and increased to 13.9 ± 3.7 pmol/d in

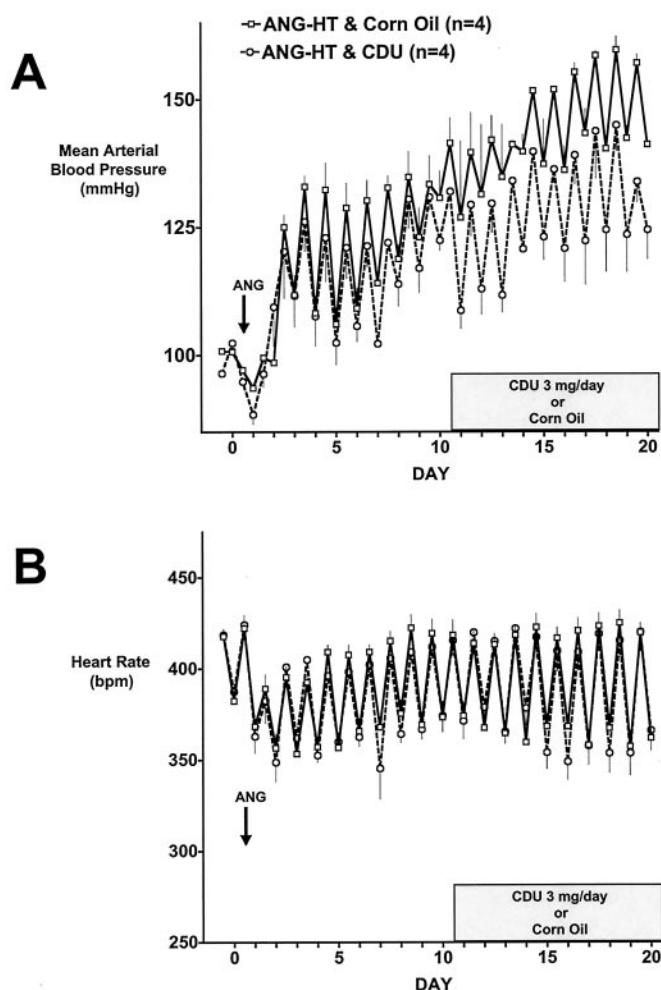


Figure 1. Effect of epoxide hydrolase inhibition on BP. Data represent day and night 12-h average BP (A) and heart rate (B) in angiotensin-treated rats with or without 1-cyclohexyl-3-dodecylurea (CDU) treatment. Angiotensin infusion was initiated on day 0 and continued to day 20. CDU or corn oil vehicle were administered from day 10 to 20. Values are mean \pm SEM.

Table 1. Plasma CYP450 metabolites in control, angiotensin-infused, and angiotensin-infused CDU-treated rats

Plasma Oxylipid Metabolites	Control (n = 4)	Angiotensin (n = 6)	Angiotensin & CDU (n = 5)
EPOME (pmol/ml)	93.3 ± 30.9	153.6 ± 29.4	347.8 ± 107.9 ^a
DHOME (pmol/ml)	25.7 ± 7.9	44.5 ± 11.6	17.7 ± 3.8 ^a
EPOME:DHOME Ratio	3.6	3.5	19.6
EET (pmol/ml)	72.9 ± 8.6	61.3 ± 3.6	67.4 ± 6.9
DHET (pmol/ml)	15.6 ± 1.27	12.6 ± 1.3	10.5 ± 0.7
EET:DHET Ratio	4.7	4.9	6.4
12,13-EPOME	86 ± 1%	88 ± 1%	89 ± 1%
9,10-EPOME	14 ± 1%	12 ± 1%	11 ± 1%
12,13-DHOME	64 ± 5%	70 ± 2%	53 ± 3%
9,10, DHOME	36 ± 5%	30 ± 2%	47 ± 3%
14,15-EET	71 ± 1%	68 ± 2%	59 ± 9%
11,12-EET	10 ± 1%	10 ± 1%	13 ± 3%
8,9-EET	13 ± 1%	16 ± 1%	23 ± 6%
5,6-EET	7 ± 1%	6 ± 1%	6 ± 1%
14,15-DHET	17 ± 1%	16 ± 1%	11 ± 1%
11,12-DHET	14 ± 1%	13 ± 1%	10 ± 1%
8,9-DHET	6 ± 1%	6 ± 1%	6 ± 1%
5,6-DHET	63 ± 1%	65 ± 1%	73 ± 2%

Values are mean ± SE. % indicates the percentage of specific EPOME, DHOME, EET, and DHET in total EPOME, DHOME, EET, and DHET, respectively.

^a Significant difference when compared to angiotensin infused rats ($P < 0.05$).

Table 2. Urinary CYP450 metabolites in control, angiotensin-infused, and angiotensin-infused CDU-treated rats

Urinary Oxylipid Metabolites	Control (n = 4)	Angiotensin (n = 6)	Angiotensin & CDU (n = 5)
EPOME (pmol/d)	3,298 ± 654	2,475 ± 186	3,699 ± 124 ^a
DHOME (pmol/d)	1,513 ± 350	2,233 ± 394	2,797 ± 894
EPOME:DHOME Ratio	2.2	1.1	1.3
EET (pmol/d)	29.3 ± 8.41	17.6 ± 2.5	34.2 ± 7.5 ^a
DHET (pmol/d)	62.0 ± 13.1	95.4 ± 13.8	86.6 ± 26.4
EET:DHET Ratio	0.5	0.2	0.4
12,13-EPOME	77 ± 1%	79 ± 1%	71 ± 8%
9,10-EPOME	23 ± 1%	22 ± 1%	29 ± 8%
12,13-DHOME	74 ± 1%	68 ± 5%	73 ± 1%
9,10, DHOME	26 ± 1%	33 ± 5%	27 ± 1%
14,15-EET	67 ± 5%	51 ± 8%	86 ± 9%
11,12-EET	15 ± 2%	40 ± 19%	7 ± 5%
8,9-EET	18 ± 4%	9 ± 6%	7 ± 5%
5,6-EET ^b	ND	ND	ND
14,15-DHET	4 ± 1%	11 ± 4%	3 ± 1%
11,12-DHET	1 ± 1%	1 ± 1%	2 ± 1%
8,9-DHET	4 ± 1%	4 ± 2%	2 ± 1%
5,6-DHET	91 ± 1%	85 ± 2%	94 ± 2%

Values are mean ± SE. Loss of this compound is apparently due to internal cyclization to the 5,6-delta lactone, not hydrolysis to the 5,6-DHET (29). % indicates the percentage of specific EPOME, DHOME, EET, and DHET in total EPOME, DHOME, EET, and DHET, respectively.

^a Significant difference when compared to angiotensin infused rats ($P < 0.05$).

^b 5,6-EET recovers with the implemented procedure are roughly 25%.

angiotensin-infused animals. CDU treatment for 10 d decreased 14,15-DHET levels to 4.2 ± 2.0 pmol/d in angiotensin hypertension. This finding is consistent with our previously published data in angiotensin-infused rats treated with CDU for 4 d (14). Urinary EET excretion rates decreased in angiotensin-infused rats, while CDU treatment for 10 d increased the urinary EET excretion rate by 94% in angiotensin-infused rats.

We also determined the regulation of SEH protein in renal microvessels and cortex. Densitometric analysis demonstrate that renal cortical SEH expression was significantly increased threefold (86 ± 5 versus 27 ± 8 d.u.; $P < 0.05$; $n = 4$ /group), but MEH protein expression was unaltered (88 ± 8 versus 81 ± 5 d.u.; $n = 4$ /group) 3 d after the start of angiotensin infusion (Figure 2). Figure 3 presents representative Western blots of SEH and MEH in the renal microvessels 2 wk after the start of angiotensin infusion. Renal microvascular SEH protein levels were increased in rats with angiotensin hypertension (83 ± 5 versus 33 ± 7 d.u.; $P < 0.05$; $n = 4$ /group). In contrast, no significant change in MEH protein levels was observed between normotensive (38 ± 4 d.u.; $n = 4$) and angiotensin-infused hypertensive rats (38 ± 5 d.u.; $n = 4$).

The responsiveness of the afferent arteriole to angiotensin before and after acute CDU administration was assessed to investigate effect of SEH inhibition on renal vascular reactivity in angiotensin hypertension. As shown in Figure 4, afferent arteriolar diameters of normotensive animal averaged 19.2 ± 0.6 μ m. Diameters of the afferent arterioles were 14% ($P < 0.05$) smaller in rats infused with angiotensin for 2 wk. Administration of the SEH inhibitor, CDU, did not alter afferent arteriolar diameter in normotensive or hypertensive animals. Angiotensin (10 nM) decreased afferent arteriolar diameter by $51\% \pm 8\%$ ($n = 8$) in hypertensive animals. CDU treatment reduced the diameter response of the afferent arteriole to 10 nM angiotensin in the hypertensive rats. The response in the

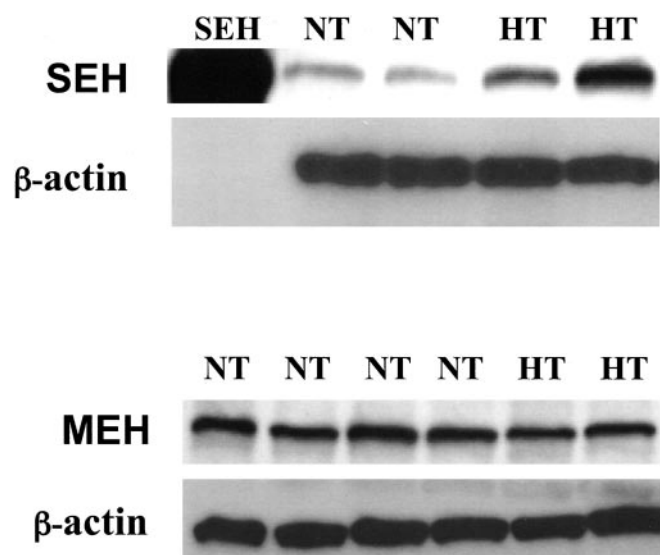


Figure 3. Renal microvessel soluble epoxide hydrolase (SEH) and microsomal epoxide hydrolase (MEH) protein expression on day 14 in normotensive control animals (NT) and angiotensin-infused animals (HT). Representative blots showing 62 kDa SEH and β -actin protein bands are shown. Recombinant SEH protein was used as a positive control for SEH (SEH, lane 1).

hypertensive afferent arterioles to angiotensin after CDU treatment was similar to that observed in normotensive animals ($28\% \pm 7\%$ versus $33\% \pm 7\%$).

Renal vascular and glomerular injury was assessed in normotensive, angiotensin-infused and CDU-treated angiotensin-infused animals. Histologic evaluation of kidneys harvested 20 d after the start of angiotensin infusion indicated that widespread renal injury characterized by focal and segmental glomerulosclerosis, glomerular hypertrophy, mesangial matrix expansion and adhesion of the glomerular tuft to Bowman's capsule is evident. In addition, in the kidneys of angiotensin-infused rats, marked tubulointerstitial damage (*i.e.*, dilated tubules, tubular cast formation, sloughing of the epithelial cells, and infiltration of mononuclear cells) could be observed. Thickening of interstitial arterial walls due to hyaline deposition and medial hypertrophy was noted (Figure 5). Semiquantitative analysis was performed in a blinded fashion and the results are presented in Table 3. Normotensive animals showed the absence of renal damage and had an injury score of zero in all categories. As expected, the renal injury in angiotensin hypertension averaged close to a +2 in all categories (Table 3). This degree of renal injury is consistent with the urinary albumin levels. Interestingly, 10 d of CDU treatment decreased the renal injury score in every category and most notable CDU administration greatly attenuated interstitial mononuclear cell infiltration. CDU-treated angiotensin-infused rats also did not have any evidence of hyaline deposition on the arterial walls.

Collagen type IV immunoreactive staining and urinary albumin levels were determined as additional parameters to assess the degree of renal damage in angiotensin hypertension. Angiotensin hypertensive rats demonstrated collagen type IV

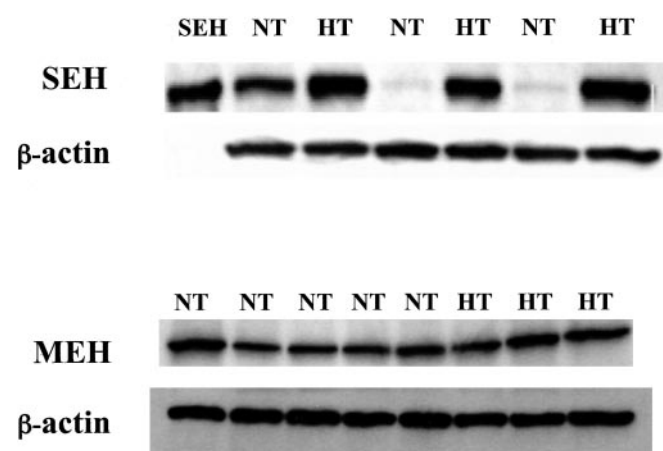


Figure 2. Renal cortical soluble epoxide hydrolase (SEH) and microsomal epoxide hydrolase (MEH) protein expression on day 3 in normotensive control animals (NT) and angiotensin-infused animals (HT). Representative blots showing 62 kDa SEH and β -actin protein bands are shown. Recombinant SEH protein was used as a positive control for SEH (lane 1). We have previously shown that the MEH antibody fails to recognize the recombinant SEH protein (15).

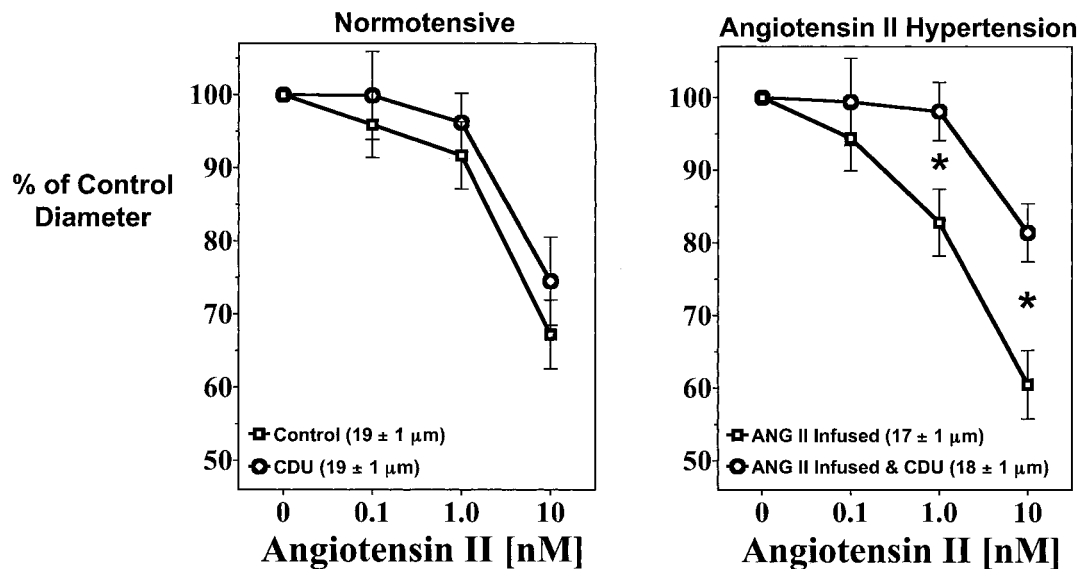


Figure 4. Effect of 1-cyclohexyl-3-dodecylurea (CDU) administration on the afferent arteriolar diameter response to angiotensin in kidneys from normotensive and angiotensin-infused hypertensive animals. Values in parentheses represent afferent arteriolar diameter before the administration of angiotensin. Values are mean \pm SEM. *Significant difference between angiotensin-infused and angiotensin- and CDU-treated group.

accumulation in glomeruli and epithelial cells throughout the kidney cortex (Figure 6). Ten days of CDU treatment to angiotensin-infused rats greatly decreased collagen IV accumulation in the glomeruli and epithelial cells. Albumin excretion rates were also measured as an index of renal damage. Urinary albumin excretion rates averaged 0.6 ± 0.1 mg/d in control animals and were elevated in angiotensin hypertension (8.2 ± 0.1 mg/d). Ten days of CDU treatment significantly decreased urinary albumin excretion in angiotensin-infused rats (3.1 ± 0.4 mg/d). Overall, the histologic findings and urinary albumin excretion rates suggest that CDU treatment ameliorated kidney damage induced by long-term continuous administration of angiotensin.

Discussion

It has been recently proposed that SEH inhibitors have striking influences on inflammation, hypertension, and vascular smooth muscle cell proliferation (14,16,17). Our previous study indicated that renal cortical SEH protein is increased in angiotensin hypertension, and chronic administration of the highly selective SEH inhibitor CDU (a.k.a. NCND) increased EET levels and decreased arterial BP in angiotensin hypertension (14). The study presented here extends these findings to explore the influence of longer term SEH inhibition on renal vascular function and vascular and glomerular injury induced by angiotensin infusion. The results of these experiments demonstrate that renal microvascular SEH protein is increased in angiotensin hypertension. In agreement with the increased SEH levels in renal microvessels, administration of CDU attenuated the afferent arteriolar response to angiotensin in the angiotensin hypertensive rats. In addition, chronic administration of the CDU lowered BP and ameliorated renal damage associated with angiotensin hypertension.

Epoxides are converted to their corresponding diols by epoxide hydrolase enzymes. In the case of arachidonic epoxygenases, EET dilate the renal vasculature whereas their corresponding DHET are less active or lack vascular activity (3,22,23). Thus, increased conversion of EET to their corresponding diols could reduce their antihypertensive, anti-inflammatory, and antimigratory attributes. Our current studies showed that both MEH and SEH enzymes were expressed in renal microvessels. Although renal microvascular MEH protein levels were not altered, angiotensin hypertension did increase SEH levels in renal microvessels. The ability of CDU to lower BP in angiotensin hypertension confirms that kidney SEH plays a critical role in the development of hypertension.

The present findings also suggest that SEH contributes significantly to the regulation of renal vascular resistance in angiotensin hypertension. Angiotensin hypertensive animals have higher SEH protein levels in renal microvessels. A number of studies by others and our laboratory have demonstrated that angiotensin hypertension is associated with an increased preglomerular resistance and an enhanced reactivity that is selective for angiotensin (24–26). Other animal models of hypertension and human patients with hypertension also exhibit enhanced angiotensin vascular reactivity (27–29). We have also demonstrated that acute elevation of 11,12-EET levels ameliorated the enhanced afferent arteriolar responsiveness to angiotensin in hypertension (30). This finding suggests that angiotensin coupling to vascular EET production may be impaired in angiotensin hypertension. In the study presented here, we present evidence that increased SEH levels may result in increased EET hydration to their less active DHET. Afferent arteriolar functional responses in angiotensin hypertension before and after CDU treatment provide further support for this concept. Like increasing 11,12-EET levels, SEH inhibition

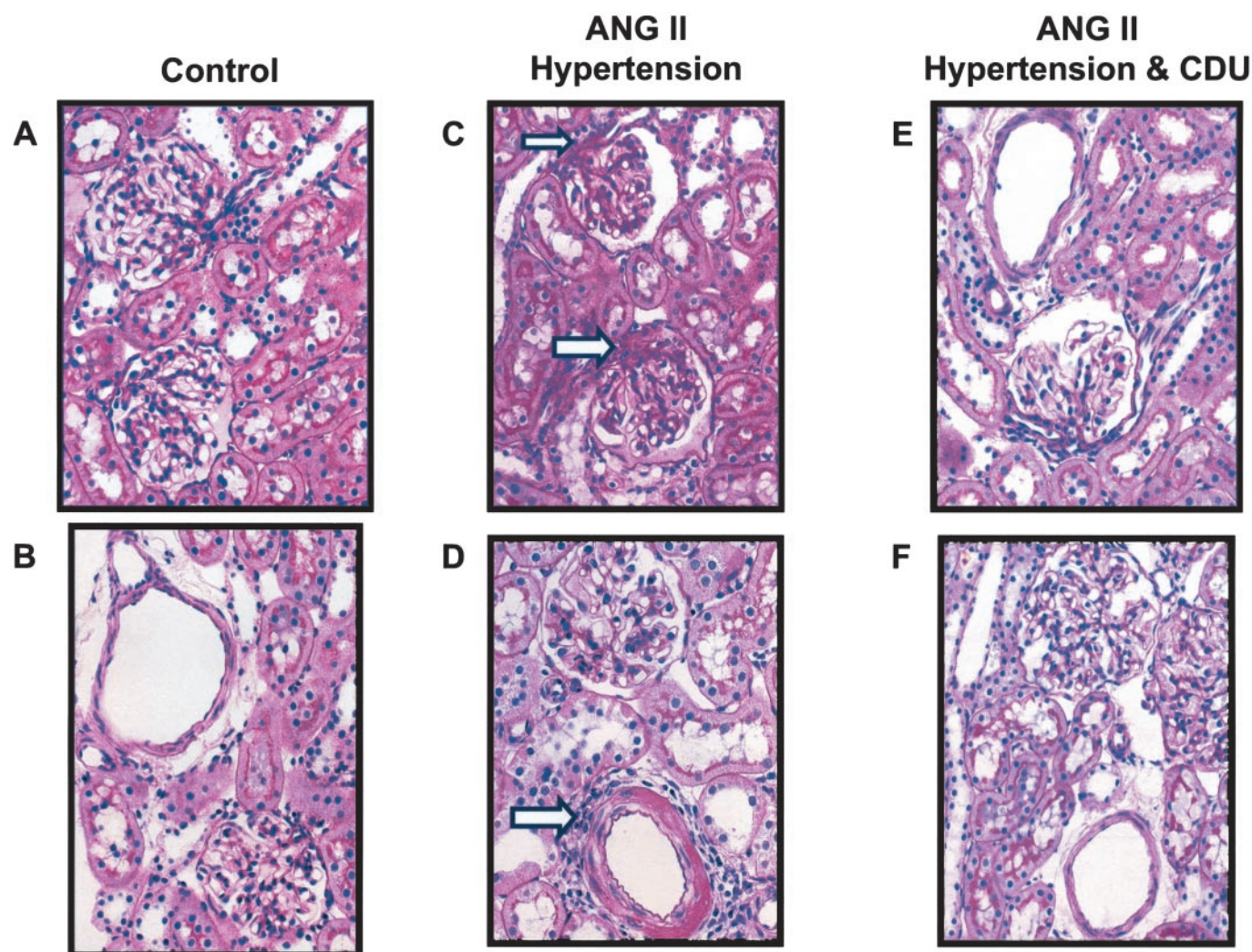


Figure 5. Comparison of morphologic appearance of paraffin-embedded kidney sections stained with periodic acid–Schiff in control (A, B), angiotensin-infused (C, D), and 1-cyclohexyl-3-dodecylurea (CDU)-treated angiotensin-infused (E, F) groups. The arrow in Panel C shows segmental glomerulosclerosis. (D) Vascular wall thickening due to hyaline deposition (arrow) and medial hypertrophy. Focal interstitial mononuclear cell infiltration was also noted.

could attenuate afferent arteriolar responses to angiotensin in angiotensin hypertension. Thus, SEH inhibition counteracts the deleterious actions of angiotensin hypertension on afferent arteriolar function.

The effect of long-term treatment with an SEH inhibitor on BP was assessed in angiotensin hypertension. Administration of CDU for 10 d significantly lowered BP in angiotensin-infused rats. These data are consistent with a previous report

from our laboratory demonstrating that 4 d of CDU lowered BP and increased urinary EET levels in angiotensin hypertension (14). Evidence that SEH is involved in maintenance of BP regulation was demonstrated in SEH gene disrupted mice that have decreased BP (31). A central role for the SEH enzyme in the pathogenesis of hypertension in the spontaneously hypertensive rat (SHR) has also been established (16,32). Yu *et al.* (16) demonstrated that a single dose of N,N'-dicyclohexylurea

Table 3. Semiquantitative histological grading in angiotensin-infused and angiotensin-infused CDU treated rats

	Mesangial Proliferation	Mesangial Expansion	Interstitial Mononuclear Cell Infiltration	Arteriolar Intimal Thickening
Angiotensin (<i>n</i> = 5)	+2.0 ± 0.0	+1.8 ± 0.2	+2.0 ± 0.3	+1.8 ± 0.2
Angiotensin & CDU (<i>n</i> = 5)	+1.6 ± 0.2	+1.3 ± 0.2 ^a	+0.4 ± 0.2 ^a	+1.3 ± 0.2 ^a

^a Significant difference between angiotensin-infused and angiotensin and CDU-treated group.

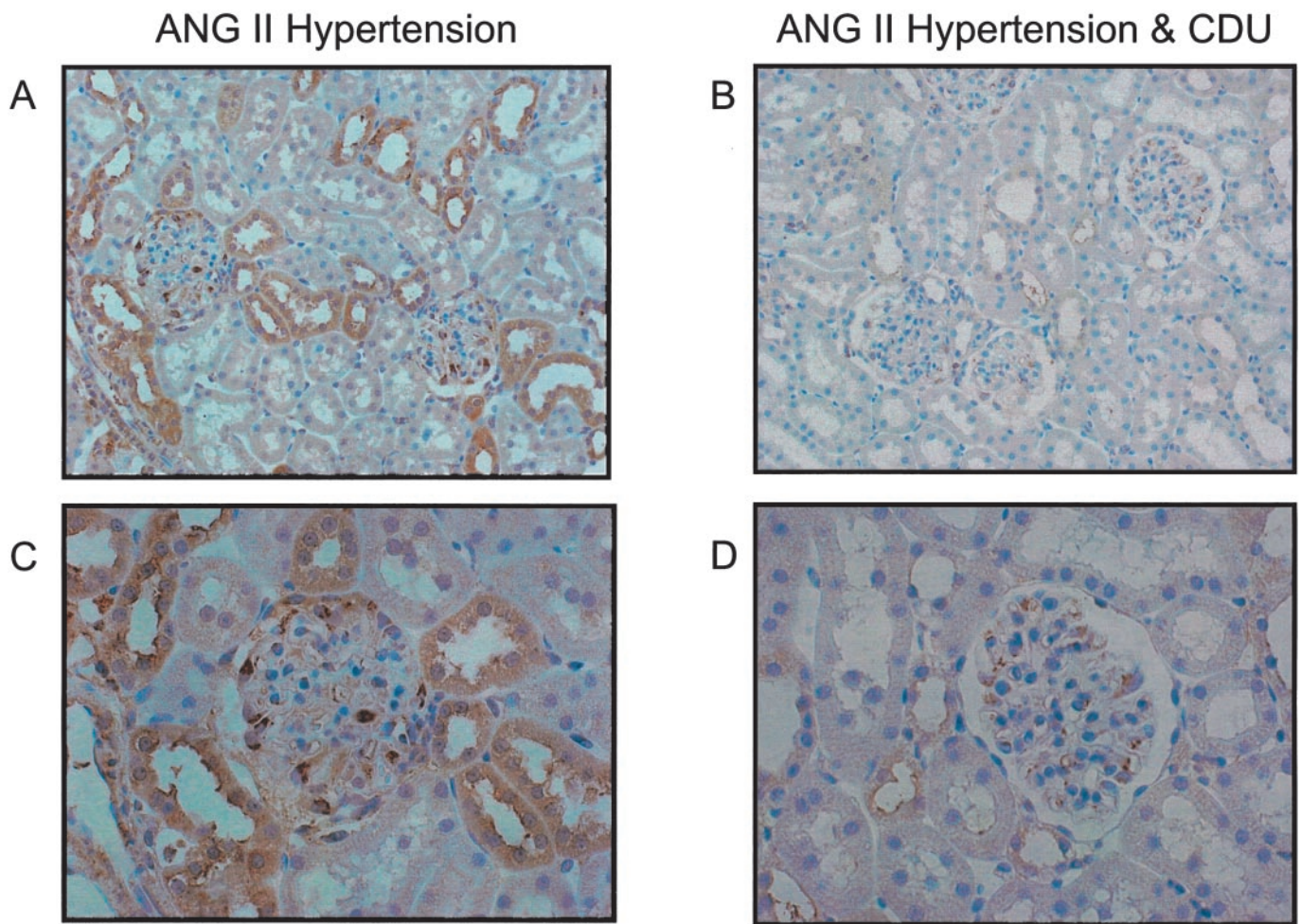


Figure 6. Collagen type IV expression in angiotensin-infused and 1-cyclohexyl-3-dodecylurea (CDU)-treated angiotensin-infused groups. Immunohistochemistry demonstrates collagen type IV expression in glomeruli and tubular epithelial cells of angiotensin-infused rats (a, low magnification 10 \times objective; c, high magnification 20 \times objective). Collagen type IV is weakly expressed in angiotensin-infused rats treated with CDU for 10 d (b, low magnification 10 \times objective; d, high magnification 20 \times objective).

(DCU) decreased BP and urinary DHET excretion in the SHR. The effect of DCU on BP in the SHR lasted less than 24 h (16). In the study presented here we demonstrate that administration of an SEH inhibitor over a 10-d period does not lower BP beyond what is observed in the first 4 d. This finding would suggest that chronic treatment of hypertension with CDU will not lower BP to levels observed in control rats.

We conducted additional studies to evaluate the circulating levels of CDU obtained by our treatment and to assess its ability to increase EET levels. We were able to measure CDU and metabolites in the urine and plasma of the angiotensin-infused animals administered CDU. CDU and the hydroxylated metabolite CHDU were the primary plasma constituents found with CHDU at a higher concentration. In contrast, CDU was the main constituent of the urine and CDU metabolites were excreted at low levels. On the basis of these measurements, it is estimated that the CDU plasma concentration was approximately 10 μ M, whereas CDU had an average concentration of 32 μ M in the urine. These findings confirm the ability of CDU to reach circulating levels necessary for inhibition of the SEH enzyme (15,33).

To further substantiate the efficacy of CDU treatment, we measured urine and plasma arachidonic and linoleic acid metabolites. The linoleic acid epoxygenase metabolites, EPOME, increased in plasma and urine and plasma DHOME decreased at the end of the 10-d CDU treatment of angiotensin hypertensive rats. One cannot rule out the possible contribution of these linoleic acid metabolites to ameliorate renal injury. To date, very little is known about the renal and cardiovascular actions of EPOME and DHOME. 12,13-EPOME and 12,13-DHOME have positive inotropic actions on the isolated rat heart (34). In addition, EPOME infused into conscious rats demonstrated a slight decline in BP but no change in heart rate (34). The renal and cardiovascular actions of the linoleic acid metabolites and their contribution to the effects of SEH inhibition await further exploration. A significant finding of this study is that the measurement of urinary arachidonic acid and linoleic acid metabolites and epoxide to diol ratios appears to be a better indicator of kidney SEH activity. Plasma arachidonic and linoleic acid metabolites as measured in this study may not be a good indicator of SEH activity because total metabolite levels were determined. Nevertheless, consistent with our previous

report (14), urinary EET levels were increased two fold and 14,15-DHET levels decreased in angiotensin-infused animals treated with CDU. These findings support the notion that inhibition of the SEH enzyme is likely responsible for the renal and cardiovascular functional changes produced by CDU treatment.

Renal vascular injury was assessed in angiotensin-infused animals treated for 10 d with CDU. Attenuation of renal vascular hypertrophy and glomerular injury was observed in CDU-treated angiotensin hypertensive rats. Increased collagen type IV expression was observed in the glomeruli and tubular epithelial cells of angiotensin-infused rats and deposition of collagen was greatly attenuated by 10 d of CDU treatment. In addition, urinary albumin levels were decreased by CDU treatment and provide additional evidence that end organ damage was decreased in hypertensive animals treated with CDU. Angiotensin infusion in rats has been demonstrated to induce both vascular and tubulointerstitial injury in kidneys (35). Although controversial, clinical studies have found that despite identical reductions in BP, combined angiotensin-converting enzyme (ACE) inhibition and angiotensin type I receptor blocker (ARB) treatment in patients with nephropathy was more effective than either agent alone (36,37). Interestingly, the added beneficial renal and cardiovascular actions of ACE inhibition have been attributed to increased kinin and EDHF levels (38–40). Renal damage has also been linked to kidney specific down regulation of epoxygenase enzymes in the dTGR hypertensive rat and suggests that increasing EET levels may have renal protective actions (41). Our current findings support the notion that increasing EET levels provides protection from end organ damage associated with hypertension. A number of studies have demonstrated anti-inflammatory and cardiovascular protective actions of EET (9–11,42,43). It should be pointed out that the renal protective effects of CDU treatment occurred with a moderate decrease in BP. The effect of the decrease in BP *versus* other aspects of CDU treatment on renal injury is not known. Taken as a whole, there is ample evidence to support the proposition that increasing EET levels provides renal and cardiovascular protection from hypertension induced end organ damage.

In addition to hypertension, increased arterial pressure is a complicating factor in numerous clinically relevant conditions including diabetes, polycystic kidney disease, and pregnancy (44,45). End organ damage associated with renal and cardiovascular diseases is a major cause of morbidity and mortality. On the basis of a number of recent studies, we tested the postulate that increasing EET levels *in vivo* through inhibition of SEH is a promising end organ damage therapeutic target. In the study presented here, we demonstrate that chronic administration of the SEH inhibitor CDU lowers arterial BP in angiotensin-dependent hypertension. More importantly, we demonstrated that CDU increased EET and EPOME levels and provided added beneficial protection to the kidney. Therefore, the modulation of endogenous lipid epoxides by SEH inhibitors may have therapeutic benefits for hypertension and end organ damage.

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References

1. Zeldin DC: Epoxygenase pathways of arachidonic acid metabolism. *J Biol Chem* 276: 36059–36062, 2001
2. Campbell WB: New role for epoxyeicosatrienoic acids as anti-inflammatory mediators. *TIPS* 21: 125–127, 2000
3. Imig JD: Eicosanoid regulation of the renal vasculature. *Am J Physiol Renal Physiol* 279: F965–F981, 2000
4. Roman RJ: P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol Rev* 82: 131–185, 2002
5. Capdevila JH, Falck JR: The CYP P450 arachidonic acid monooxygenases: From cell signaling to blood pressure regulation. *Biochem Biophys Res Commun* 285: 571–576, 2001
6. Imig JD, Falck JR, Wei S, Capdevila JH: Epoxygenase metabolites contribute to the nitric oxide-independent afferent arteriolar vasodilation to bradykinin. *J Vasc Res* 38: 247–255, 2001
7. Campbell WB, Gebremedhin D, Pratt PF, Harder DR: Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res* 78: 415–423, 1996
8. Fissthaler B, Hinsch N, Chataigneau T, Popp R, Kiss L, Busse R, Fleming I: Nifedipine increases cytochrome P4502C expression and endothelium-derived hyperpolarizing factor-mediated responses in coronary arteries. *Hypertension* 36: 270–275, 2000.
9. Node K, Ruan XL, Dai J, Yang SX, Graham LR, Zeldin DC, Liao JK: Activation of G α_s mediates induction of tissue-type plasminogen activator gene transcription by epoxyeicosatrienoic acids. *J Biol Chem* 276: 15983–15989, 2001
10. Node K, Huo Y, Ruan X, Yang B, Spieker M, Ley K, Zeldin DC, Liao JK: Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science* 285: 1276–1279, 1999
11. Sun J, Sui XX, Bradbury A, Zeldin DC, Conte MS, Liao JK: Inhibition of vascular smooth muscle cell migration by cytochrome P450 epoxygenase-derived eicosanoids. *Circ Res* 90: 1020–1027, 2002
12. Zhao X, Pollock DM, Inscho EW, Zeldin DC, Imig JD: Decreased renal CYP2C enzymes and impaired vasodilation are associated with salt-sensitive hypertension. *Hypertension* 41: 709–714, 2003
13. Makita K, Takahashi K, Karara A, Jacobson HR, Falck JR, Capdevila JH: Experimental and/or genetically controlled alterations of the renal microsomal cytochrome P450 epoxygenase induce hypertension in rats fed a high salt diet. *J Clin Invest* 94: 2414–2420, 1994
14. Imig JD, Zhao X, Capdevila JH, Morisseau C, Hammock BD: Soluble epoxide hydrolase inhibition lowers arterial blood pressure in angiotensin II hypertension. *Hypertension* 39: 690–694, 2002
15. Morisseau C, Goodrow MH, Dowdy D, Zheng J, Greene JF, Sanborn JR, Hammock BD: Potent urea and carbamate inhibitors of soluble epoxide hydrolases. *PNAS USA* 96: 8849–8854, 1999
16. Yu Z, Xu F, Huse LM, Morisseau C, Draper AJ, Newman JW, Parker C, Graham L, Engler M, Hammock BD, Zeldin DC,

- Kroetz DL: Soluble epoxide hydrolase regulates hydrolysis of vasoactive epoxyeicosatrienoic acids. *Circ Res* 87: 992–998, 2000
17. Davis BB, Thompson DA, Howard LL, Morisseau C, Hammock BD, Weiss RH: Inhibitors of soluble epoxide hydrolase attenuate vascular smooth muscle cell proliferation. *PNAS USA* 99: 2222–2227, 2002
18. Sasser JM, Pollock JS, Pollock DM: Renal endothelin in chronic angiotensin II hypertension. *Am J Physiol Regulatory Integrative Comp Physiol* 283: R243–R248, 2002
19. Newman JW, Wantanabe T, Hammock BD: The simultaneous quantification of cytochrome P450 dependent linoleate and arachidonate metabolites in urine by HPLC-MS/MS. *J Lip Res* 43: 1563–1578, 2002
20. Watanabe T, Morisseau C, Newman JW, Hammock BD: In vitro metabolism of the mammalian soluble epoxide hydrolase inhibitor, 1-cyclohexyl-3-dodecyl-urea. *Drug Metab Dispos* 31: 846–853, 2003
21. Zou LX, Imig JD, Von Thun AM, Hymel A, Ono H, Navar LG: Receptor-mediated intrarenal angiotensin II augmentation in angiotensin II-infused rats. *Hypertension* 28: 669–677, 1996
22. Imig JD, Navar LG, Roman RJ, Reddy KK, Falck JR: Actions of epoxigenase metabolites on the glomerular vasculature. *J Am Soc Nephrol* 7: 2364–2370, 1996
23. Zou AP, Fleming JT, Falck JR, Jacobs ER, Gebremedhin D, Harder DR, Roman RJ: Stereospecific effects of epoxyeicosatrienoic acids on renal vascular tone and K⁺ channel activity. *Am J Physiol Renal Physiol* 270: F822–F832, 1996
24. Imig JD: Afferent arteriolar reactivity to angiotensin II is enhanced during the early phase of angiotensin II hypertension. *Am J Hypertension* 13: 810–818, 2000
25. Simon G, Abraham G: Angiotensin II administration as an experimental model of hypertension. In: *Hypertension: Pathophysiology, Diagnosis, and Management*, edited by Laragh JH, Brenner BM, New York, Raven Press, 1995, pp 1423–1435.
26. Stevenson KM, Edgley AJ, Bergstrom G, Worthy K, Kett MM, Anderson WP: Angiotensin II infused intrarenally causes preglomerular vascular changes and hypertension. *Hypertension* 36: 839–844, 2000
27. Chatziantoniou C, Daniels FH, Arendshorst WJ: Exaggerated renal vascular reactivity to angiotensin and thromboxane in young genetically hypertensive rats. *Am J Physiol Renal Physiol* 259: F372–F382, 1990
28. Ploth DW, Fitzgibbon W: Pathophysiology of altered renal function in renal vascular hypertension. *Am J Kidney Dis* 24: 652–659, 1994
29. Mulvany MJ: Structural changes in the resistance vessels in human hypertension. In: *Hypertension: Pathophysiology, Diagnosis, and Management*, edited by Laragh JH, Brenner BM, New York, Raven Press, 1995, pp 503–513.
30. Imig JD, Falck JR, Wei S, Capdevila JH: Enhanced renal microvascular responsiveness to angiotensin II in hypertension is ameliorated by the sulfonimide analog of 11,12-epoxyeicosatrienoic acid. *J Hypertens* 19: 983–992, 2001
31. Sinal CJ, Miyata M, Tohkin M, Nagata K, Bend JR, Gonzalez FJ: Targeted disruption of soluble epoxide hydrolase reveals a role in blood pressure regulation. *J Biol Chem* 275: 40504–40510, 2000
32. Fornage M, Hinojos CA, Nurowska BW, Boerwinkle E, Hammock BD, Morisseau C, Doris PA: Polymorphism in soluble epoxide hydrolase and blood pressure in spontaneously hypertensive rats. *Hypertension* 40: 485–490, 2002
33. Morisseau C, Goodrow MH, Newman JW, Wheelock CE, Dowdy DL, Hammock BD: Structural refinement of inhibitors of urea-based soluble epoxide hydrolases. *Biochem Pharmacol* 63: 1599–1608, 2002
34. Mitchell LA, Grant DF, Melchert RB, Petty NM, Kennedy RH: Linoleic acid metabolites act to increase contractility in isolated rat heart. *Cardiovasc Toxicol* 2: 219–230, 2002
35. Lombardi DM, Viswanathan M, Vio CP, Saavedra JM, Schwartz SM, Johnson RJ: Renal and vascular injury induced by exogenous angiotensin II is AT1 receptor-dependent. *Nephron* 87: 66–74, 2001
36. Campbell R, Sangalli F, Perticucci E, Aros C, Viscarra C, Perna A, Remuzzi A, Bertocchi F, Fagiani L, Remuzzi G, Ruggerenti P: Effects of combined ACE inhibitor and angiotensin II antagonist treatment in human chronic nephropathies. *Kidney Int* 63: 1094–1103, 2003
37. Russo D, Pisani A, Balletta MM, De Nicola L, Savino FA, Andreucci M, Minutolo R: Additive antiproteinuric effect of converting enzyme inhibitor and losartan in normotensive patients with IgA nephropathy. *Am J Kidney Dis* 33: 851–856, 1999
38. Matsuda H, Hayashi K, Arakawa K, Naitoh M, Kubota E, Honda M, Matsumoto A, Suzuki H, Yamamoto T, Kajiya F, Saruta T: Zonal heterogeneity in action of angiotensin-converting enzyme inhibitor on renal microcirculation: Role of intrarenal bradykinin. *J Am Soc Nephrol* 10: 2272–2282, 1999
39. Fenoy FJ, Scicli G, Carretero O, Roman RJ: Effect of angiotensin II and a kinin receptor antagonist on the renal hemodynamic response to captopril. *Hypertension* 17: 1038–1044, 1991
40. Goto K, Fujii K, Onaka U, Abe I, Fujishima M: Renin-angiotensin system blockade improves endothelial dysfunction in hypertension. *Hypertension* 36: 575–580, 2000
41. Kaergel E, Muller DN, Honeck H, Theuer J, Shagdarsuren E, Mullally A, Luft FC, Schunck WH: P450-dependent arachidonic acid metabolism and angiotensin II-induced renal damage. *Hypertension* 40: 273–279, 2002
42. Kozak W, Klugar MJ, Kozak A, Wachulec M, Dokladny K: Role of cytochrome P-450 in endogenous antipyreresis. *Am J Physiol Regul Integr Comp Physiol* 279: R455–R460, 2000
43. Nakashima T, Yoshida Y, Miyata S, Kiyohara T: Hypothalamic 11,12-epoxyeicosatrienoic acid attenuates fever induced by central interleukin 1 β in the rat. *Neurosci Lett* 310: 141–144, 2001
44. Crook ED: The role of hypertension, obesity, and diabetes in causing renal vascular disease. *Am J Med Sci* 317: 183–188, 1999
45. Brenner BM: The epidemic of cardiovascular disease in end-stage renal disease. *Curr Opin Nephrol Hypertens* 8: 365–369, 1999